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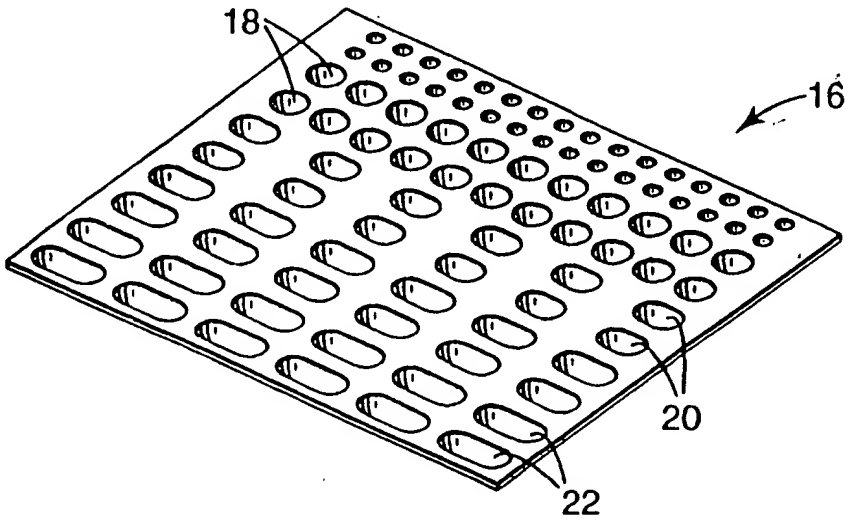

(57) Abstract

A method for detecting a microorganism in a test sample is described. The method involves distributing microvolumes 0.01-25 microlitres of a sample to a plurality of microcompartments of a culture device, incubating for a time sufficient to permit at least one cell division cycle of the microorganism, then detecting the presence or absence of the microorganism in the microcompartments. Also disclosed are devices for carrying out these methods.

METHOD AND DEVICES FOR DETECTING AND ENUMERATING MICROORGANISMS

This application is a continuation-in-part of Application No. 08/838,552 filed April 9, 1997.

This invention relates to methods and devices that use microvolume compartments to effect rapid and accurate detection and enumeration of microorganisms.

The detection and enumeration of microorganisms is practiced in numerous settings, including the food-processing industry (testing for the contamination of food by microorganisms such as *E. coli* and *S. aureus*), the health care industry (testing of patient samples and other clinical samples for infection or contamination), environmental testing industry, pharmaceutical industry, and cosmetic industry.

Growth-based detection and enumeration of microorganisms is commonly practiced using either liquid nutrient media (most probable number analysis (MPN)) or semi-solid nutrient media (direct counting using, e.g., agar petri dishes). Enumeration using the liquid MPN method is typically achieved by placing serial 10-fold dilutions of a sample of interest in replicate sets of tubes containing selective media and chemical indicators. The tubes are incubated at elevated temperature (24-48 hours) followed by examination for organism growth. A statistical formula, based on the number of positive and negative tubes for each set, is used to estimate the number of organisms present in the initial sample.

This method of performing MPN analysis has several disadvantages. It is labor intensive because of the multiple diluting and pipetting steps necessary to perform the analysis. In addition, it is only practical to use replicate sets of about three to five tubes for each dilution. As a result, the 95% confidence limits for an

distributing microvolumes of the sample to a plurality of microcompartments of a culture device; incubating the culture device for a time sufficient to permit at least one cell division cycle of the microorganism; and detecting the presence or absence of the microorganism in the microcompartments.

As used herein, the term microvolume refers to a volume of between about 0.01 and about 25 microliters, and the term "microcompartment" refers to a compartment having a capacity, or volume, to hold a microvolume of liquid test sample.

In preferred embodiments, the method further includes the step of quantitating the microorganisms in the liquid test sample. The quantitation may include the steps of determining MPN in the sample, or it may involve enumerating the microorganisms in each microcompartment of the culture device.

In other embodiments, the microcompartments may contain a coating of nutrient medium, and the nutrient medium may further include at least one indicator substance. Alternatively, the liquid test sample may include at least one indicator substance. In either case, the indicator substance may be any indicator substance capable of providing a detectable signal in the liquid test sample. Such indicators include, but are not limited to, chromogenic indicators, fluorescent indicators, luminescent indicators, and electrochemical indicators. For purposes of this application, the term "electrochemical" means a chemical indicator that changes the resistance or conductance of the sample upon reaction with a microorganism.

In another aspect, the invention features a method for detecting a microorganism in a liquid test sample. This method involves the steps of:

distributing aliquots of the sample to a plurality of microcompartments of a culture device, wherein the culture device contains a plurality of sets of microcompartments, each set having microcompartments of uniform size and the sets varying in microcompartment size; incubating the culture device for a time sufficient to permit at least one cell division cycle of the

The microcompartments can be arranged in substantially parallel rows. Typically, the volumes of the microcompartments in each row are uniform. Alternatively, the microcompartments can be arranged in various groupings or patterns for easier recognition and counting of positive signals.

The volumes of the microcompartments may range from about 0.01 to about 25 microliters, more preferably from about 0.1 to about 10 microliters, and most preferably from about 1 to about 2 microliters.

As described herein, the present invention has several advantages. First, use of microvolumes in microcompartments allows for a surprisingly rapid detection of a microorganism in a liquid test sample. Second, this rapid detection allows for rapid enumeration or quantitation of microorganisms in the liquid test sample. The invention is particularly useful in MPN analysis of a liquid test sample for a particular microorganism, such as *E. coli* or *S. aureus*. The invention allows MPN analysis to be conducted conveniently in a single device, as opposed to separate tubes, and advantageously requires a substantively shorter incubation time to reach detectable microorganism growth. Third, the use of microvolumes in microcompartments allows for the separation of a liquid test sample into a relatively larger number of test volumes. In general, the use of microvolumes in microcompartments provides a far greater number of runs, or repetitions, of a test on the liquid sample. In the case of MPN analysis, use of microvolumes in microcompartments provides a greater number of data points from which the MPN can be calculated, thereby significantly narrowing the 95% confidence limits for a given MPN result. Fourth, separation of sample into a large number of test volumes allows a higher concentration of microorganisms to be enumerated, thereby reducing or eliminating sample dilutions. Fifth, this invention allows MPN analysis to be conducted in a single device having the indicators and/or nutrients directly coated thereon. Sixth, this invention permits a wide counting range when performing MPN analysis.

Figure 1 is a perspective view of one embodiment of a microcompartment culture device.

addition, the use of a relatively large number of microvolume compartments significantly narrows the 95% confidence limits for the result and reduces the number of sample dilutions for concentrated samples.

In addition to the above advantages, the use of microvolumes in the testing of liquid samples may allow for the use of substantially smaller test samples. Very small volume test samples are sometimes necessary due to very small volume sample sources or desirable for purposes such as ease of handling.

The present inventors have developed a number of novel devices for microvolume-based testing of liquid samples. Non-limiting examples of these devices include a substrate, such as micro-embossed or pressed films having a plurality of microcompartments and having various surface treatments to improve performance and convenience and micro-embossed or pressed films having a plurality of open-bottomed microcompartments, wherein each well bottom opening is occluded by a material that is permeable to air but is substantially non-permeable to aqueous fluids. The open bottomed configuration may help to eliminate the potential problem of air bubbles being entrapped in the sample in the microcompartments.

When viewed in a top view, the microcompartment may have, for example, a generally circular, faceted, square, oval, or elongate appearance. It will be appreciated that the microcompartments of these devices may have many possible shapes, such as cylindrical, conical, pyramidal, hemispherical, tetrahedral, cubic, truncated shapes, and the like, with open or closed bottoms.

Another example includes a substrate, such as a plastic film containing microchannels, wherein the liquid sample may move into the microchannels by capillary action. The microchannels may be discrete capillary tubes that are formed or bonded together into a substrate. The cross section of each channel may take many shapes, including circular, triangular, square, and rectangular shapes and the like. In a preferred embodiment, the cross section of the end(s) of the microchannels is smaller than the cross section of the middle of the microchannels. In this configuration, sample is less likely to spill out during handling of the devices.

microliters. The culture device preferably contains about 1 to about 100,000 microcompartments, more preferably about 100 to about 10,000 microcompartments, even more preferably about 200 to about 5,000 microcompartments and most preferably about 400 to about 600 microcompartments.

The use of a device having about 400 to about 600 microcompartments is particularly useful in the context of testing a liquid sample for microorganism concentration using MPN. Certain regulatory requirements may dictate that a testing method must be able to detect one microorganism in a one to five milliliter sample. Such a sample size is standard in the food processing industry for microbiological testing. Thus, for example, a culture device having 500 microcompartments, wherein each microcompartment has a volume of about 2 microliters, would be very useful for testing a 1-ml sample. The microcompartment size of 2 microliters allows for rapid development of a detectable signal in accordance with the invention, and the use of about 400 to about 600 microcompartments provides a sufficiently large number of data points to substantially improve the confidence interval for an MPN calculation. In addition, it is feasible to perform a manual count of microcompartments testing positive for the microorganism of interest.

The liquid test sample may be any sample containing microorganisms from any source. The sample may be distributed to the plurality of microcompartments directly, or the sample may be diluted before distribution to the microcompartments. The determination as to whether sample dilution is necessary will depend on a variety of factors such as sample source and age, and such determination is a routine matter to those of skill in the art.

The liquid test sample may include selective nutrient growth media, optionally including a gelling agent, for the microorganism of interest and/or an indicator substance that produces a signal in the presence of the growing microorganism. A gelling agent is a water absorbing material that becomes a gel upon the addition of water. If a gelling agent is used, the gel preferably will encapsulate, or contain, the growing microorganism. One or both of the selective

distinct advantage over detection methods currently used, which typically require incubation times of about 24 hours or more.

Following incubation of the culture device, the presence or absence of the microorganisms in the microcompartments (and thus in the liquid test sample) is detected. The mode and sensitivity of detection depends on the type of indicator substance used in the method. In some instances, the presence or absence of the microorganism may be detected visually without the aid of signal-generating indicator substance, by visualizing the turbidity or clarity of the sample in each microcompartment. Any indicator substance that provides a detectable signal in the liquid test sample may be used, including but not limited to chromogenic indicators, fluorescent indicators, luminescent indicators, electrochemical indicators, and the like. The presence or absence of a microorganism in a microcompartment may be visually detected, with the naked eye, microscopically, or with the aid of other equipment or methods. There are numerous indicator substances and signal detection systems known in the art for detecting microorganisms, and any such substance or system may be used in accordance with the present invention.

The detection of microorganisms in the liquid sample may further involve the enumeration of a microorganism count in the liquid test sample. In a preferred embodiment, the enumeration is performed using MPN. Once the number of microcompartments containing the microorganism of interest is determined, an MPN calculation can be made using known MPN techniques. If desired, the number of microorganisms in an individual microcompartment can then be determined using known techniques, for example, signal intensity compared to a known standard, or by plating the contents of the microcompartment. Advantageously, the large number of microcompartments used in the method of the invention allows for narrower intervals for the 95% confidence limits in an MPN analysis of a liquid test sample.

Because of the large number of microcompartments in a single device that the methods and devices of the present invention provide, it is possible to use a single device in the detection and enumeration of multiple microorganisms of interest, while retaining the advantages of the invention. For example, a single

Referring to Figure 1, a device 10 may comprise a substrate 12 having a plurality of compartments in the form of microcompartments 14. The substrate 12 can be fabricated from any material in which microcompartments can be fashioned. Substrate 12 can be fabricated, for example, from polymeric films or other appropriate materials. Appropriate polymers include without limitation polyethylene, polypropylene, polyamides, fluoropolymers, polycarbonates, polyesters, polyurethanes, and polystyrenes. Microcompartments 14 can be formed by any process appropriate to the substrate 12 material. Such processes include without limitation thermal embossing, cast embossing, laser drilling, and etching with reactive materials. Alternatively, a device may be prepared by laminating a sheet of patterned material containing a plurality of small openings onto a support film, wherein a microcompartment is formed by the combination of the opening and the support film. Polyethylene or polypropylene films can be, for example, pressed embossed or extrusion embossed, and can include various pigments and surfactants.

The device 10 may include any desired number of microcompartments. Additionally, the device 10 may include relatively large reservoirs or other compartments adapted to hold larger volumes of liquid for maintenance of an appropriate humidity level within the device. Although the number of microcompartments can be relatively small (e.g., 2-50), the small sizes of the microcompartments allow relatively large numbers of microcompartments to be fabricated on a single device 10. Preferably, the device has about 1 to 100,000 microcompartments, more preferably about 100 to about 10,000 microcompartments, even more preferably between about 200 to about 5,000 microcompartments, and most preferably about 400 to about 600 microcompartments. The device 10 can have a population of uniformly sized microcompartments 14, although the microcompartments need not be of uniform size. For example, a device 16 as depicted in Figure 2 can have sets (e.g., rows) of microcompartments in which volumes are constant within a set, but vary between sets. As depicted in Figure 2, the volumes can vary incrementally over an array of sets of microcompartments, with the smaller microcompartments 18 holding sub-microliter volumes and the larger microcompartments 20 holding multiple-microliter

As discussed above, the presence of microcompartments in an assay device allows for separation of a liquid test sample into a relatively large number of test microvolumes. The ability to separate a liquid sample into microcompartments and to perform MPN or other assays without cross-contamination between compartments is a major advantage of the present devices. Various additional fabrication methods, however, can be used to further enhance the separation function of the microcompartments, as described below.

Referring again to Figure 1, the area 13 between microcompartments 14 ("land area") may be fabricated to be hydrophobic. This serves to prevent aqueous fluid from bridging between the microcompartments 14, thereby preventing cross-contamination. The land area 13 can be rendered hydrophobic in various ways. For example, the land area on an extrusion embossed polyethylene film, that had been rendered hydrophilic by incorporation of a surfactant, can be rendered hydrophobic by transferring a thin layer of acrylated silicone or other hydrophobic material to the land area.

Referring to Figure 6, the microcompartments may be fashioned as microchannels 32 in a substrate 34. The shape of the microchannel 32 may vary. The microchannel may be square bottomed, U- and V-shaped or comprise elongate holes.

Preferably, microchannel 32 is covered to prevent evaporation from the channel and contamination of the channel. The cover 36 may be prepared from any suitable material that is at least partially impermeable to water vapor. For example, the cover may comprise a silicone pressure-sensitive adhesive film or a heat sealable film.

Assay reagents may be coated into the microchannel. Preferably, at least one such reagent is coated into each microchannel.

In an alternative preferred embodiment as depicted in Figure 7, individual layers of film with microchannels 32 therein can be laminated together to form a multi-layered structure 38. This structure has many advantages, including having a large number of microcompartments in a small area and ease of innoculation of a

subsequent examples. The stacked polyethylene sheets were embossed on a heated hydraulic press (132°C, 120 second dwell) as described in U.S. Patent 5,219,462. The samples were allowed to cool, at which time the tooling was removed to provide a single layer film containing the "negative" image of the tooling.

B. Extrusion Embossed Films Containing a Plurality of Microcompartments

A sheet of photolithographically etched magnesium master tooling was attached to a steel roll using pressure-sensitive transfer adhesive. The polyethylene, pigment, and surfactant composition described in example 1A was blended together and extrusion cast onto the roll as described in U.S. Patent 5,192,548, which is incorporated herein by reference. Samples lacking the Triton X-35 were also prepared in this manner.

C. Extrusion Embossed Films With Hydrophobic "Land" Area

Extrusion embossed polyethylene films containing Triton X-35 Surfactant were prepared according to Example 1B. The area between microcompartments ("land" area) was rendered hydrophobic by transferring a thin layer of acrylated silicone (Goldschmidt FC 711) containing 4.8% of a cross linking agent (Darocur 1173) using a roll-to-roll coating apparatus (Straub Design Co.). The hydrophobic coating was cured by exposing the film to ultraviolet radiation under nitrogen atmosphere using a Fusion Systems UV lamp with an H bulb providing a dosage of 85 millijoules /cm^2. An aqueous solution containing phenol red indicator (to provide contrast) was spread over treated and untreated samples. The samples treated with the hydrophobic coating were shown to partition liquid into individual microcompartments without fluid bridging between the microcompartments.

D. Pressed Embossed Films Containing a Plurality of Microchannels

Polyethylene film (Example 1A) was cut into sheets and stacked (~10 four-mil sheets) onto magnesium tooling designed to form a plurality of parallel microchannels, followed by embossing on a heated hydraulic press according to the following protocol: heated to 143 °C, held at 0.7 N/m^2 for 1 minute, pressure increased to 2.8 N/m^2 and held for 1 minute, pressure decreased to 2.1 N/m^2 and held for .15 seconds, cooled to 29 °C, and released. The tooling was removed to

test solution and had no apparent air bubbles. In comparison, occasionally the microcompartments without perforated bottoms were observed to contain entrapped air bubbles. However, when the bottom surface of the embossed film was placed in contact with a second surface, test solution was wicked out of the microcompartments containing open bottoms. Also, during the inoculation process, it was observed that the test solution applied from a pipette at high speed sometimes leaked through the few microcompartments located directly under the tip of the pipette.

Preparation of Embossed Films With Bottom-Perforated Microcompartments Covered With Non-Woven Webs

A pressure sensitive adhesive (PSA) non-woven web material was applied to the underneath surface of the embossed polypropylene film culture devices having a plurality of bottom-perforated microcompartments in order to eliminate leaking of the test sample solution. The non-woven web was constructed of Kraton 1112 (web weight = 50 g/m^2) and contained a blown-fiber PSA as described in European Patent Application No. 94119851.7. The PSA non-woven web was easily bonded to the film by pressure and, thereby, formed a covered, but air-porous, bottom on each of the microcompartments.

Fill and Leak Testing of Films With Bottom-Perforated Microcompartments Covered With Non-Woven Webs

Butterfield diluent containing phenol red, as described above, was inoculated onto a polypropylene embossed film having a plurality of microcompartments, some of which were perforated and covered on the bottom with a non-woven web. All perforated microcompartments were filled by a simple hand-swirling of the film, and only non-perforated microcompartments entrapped air. Different amounts of test solution (1-3 ml) were applied by pipette with no leaking observed during the inoculation process. No flow of test solution through the porous, covered bottom of the microcompartments was observed under an

Example 3

Detection and Enumeration of Microorganisms

(Method Utilizing Plurality of Microcompartments)

The feasibility of utilizing embossed film culture devices containing a plurality of microcompartments to detect and enumerate *E. coli* was demonstrated in this example.

An overnight broth culture of *E. coli* ATCC 51813 (~10^9 CFU/ml in Tryptic Soy Broth (TSB) media) was serially diluted into Violet Red Bile (VRB) media (7.0 g/l Bacto peptone, 3.0 g/l yeast extract, and 1.5 g/l bile salts) containing 4-methylumbelliferyl-β-D-glucuronide (0.5 mg/ml) (MUG, Biosynth International, Naperville, IL). The dilutions were prepared to the approximate bacterial concentrations shown in Table 3a. A diluted sample (1 ml) was applied by pipette onto a polyethylene embossed film culture device (Example 1B, lacking the Triton X-35) containing 525 microcompartments (about 1.9 µl/microcompartment). The microcompartments were arranged in a hexagonal array (about 19 microcompartments /cm^2) and each microcompartment was in the shape of an inverted truncated hexagonal cone, having a diameter of approximately 1.9 mm at the surface and 1.0 mm at its depth, which was about 1.1 mm. The microcompartments were filled as described in U.S. Patent 5,219,462 by guiding the diluted sample solution down the film with the edge of a razor blade. A diluted sample (1 ml) also was placed on a PETRIFILM™ Series 2000 Rapid Coliform Test Plate (3M Company, St. Paul, MN), incubated, and read according to manufacturer's directions. The inoculated embossed film culture devices were placed inside petri dishes, and incubated for 12 hours at 37°C. The number of microcompartments exhibiting fluorescence were counted for each sample. The most probable number was calculated using the formula MPN=N ln (N/N-X) where N is the total number of filled microcompartments and X is the total number of microcompartments showing a positive reaction. The results are compared with counts from the PETRIFILM™ Series 2000 Plates in Table 3a.

web (Example 2). Each film was cut into 5.1-cm diameter circles and placed in polystyrene petri dishes (5.1-cm diameter x 1.9-cm height) for inoculation, growth and detection of bacteria. The film disc was elevated from the bottom of the petri dish by a foam spacer ring. Curable silicone was applied along the edge of the film disc edge to provide a seal between the film disc and the dish. The seal prevented test sample solutions from leaking through the edge of the film disc during inoculation. Each of the resulting culture device plates contained about 300 microcompartments (about 2.0 µl/well) and was treated by soaking in isopropanol for 2 to 3 minutes and dried overnight at ambient conditions and in an oven at 60°C for 5 minutes prior to use.

A sample (0.1 ml) from the individual dilutions was mixed with balanced aerobic count nutrient medium (0.9 ml) having the composition shown in Table 4a and containing 4-methylumbelliferylphosphate (0.1 mg/ml). The resulting test sample (~1 ml) was applied by pipette onto a culture device plate and the plate was gently agitated to deposit a portion of the sample into each of the microcompartments. The plate was then tilted to pour out the excess sample volume into an absorbent pad that was attached to the rim of the polystyrene dish. Additional distilled water (about 0.3 ml) was added to the absorbent pad to moisten it completely to provide a humidity reservoir. The inoculated plates were inverted, incubated for 24 hours at 35°C, and the number of fluorescent (positive) microcompartments counted under UV light (360 nm) excitation. Most Probable Number (MPN) was calculated according to Example 3. The MPN/ml was calculated by multiplying the MPN by 1.66 based on a sampled volume of 600 microliters contained within the 300 microcompartments of the device. Results are provided in Table 4b.

Table 4a
Balanced Aerobic Count Nutrient Medium

Ingredient	Concentration (g/l)
Sodium Pyruvate (Sigma Chem Co., St. Louis, MO)	4.4
Tryptone (Difco Labs, Detroit , MI)	7.5

Diluted samples of *E. coli* ATCC 51813 were prepared according to Example 3. Two embossed film culture devices were prepared according to Example 1B. The first contained a square array of 0.8-µl microcompartments (~16 microcompartments/cm^2), each microcompartment in the shape of an inverted truncated cone, having a diameter of approximately 1.2 mm at the surface and 0.7 mm at its depth, which was about 1.0 mm. The second film contained a square array of larger 5-µl microcompartments (~4 microcompartments/cm^2) , each microcompartment in the shape of a truncated square pyramid, having an opening of 3.7 x 3.7 mm at the surface and 2.0 x 2.0 mm at its depth, which was about 1.0 mm. A 250-µl sample of each dilution was partitioned into the microcompartments by using the procedure described in Example 3. The devices were incubated overnight at 37°C and the number of microcompartments exhibiting fluorescence was counted for each set of films. MPN values were calculated as described in Example 3. The MPN per milliliter was calculated by multiplying the value obtained for the 250-µl inoculum by 4. Results are provided in Table 5a and are compared with counts obtained from standard testing with PETRIFILM™ Series 2000 Count Plates.

Table 5a

Enumeration of Microorganisms (*E. coli*)

Final Dilution	Positive 0.8-µl Microcom-partments	MPN/ml (0.8µl)	Positive 5-µl Microcom-partments	MPN/ml (5 µl)	PETRIFILM™ Series 2000 Plates
1×10^{-5}	300	>6844	50	>782	TNTC
5×10^{-6}	300	>6844	50	>782	TNTC
1×10^{-6}	300	>6844	50	>782	TNTC
1×10^{-7}	292	4,349	50	>782	TNTC
5×10^{-8}	64	286	41	342	213
2.5×10^{-8}	36	155	31	193	136

The results of this example show that microorganisms can be readily detected and enumerated using an embossed film culture device having a plurality of microcompartments of different sets and that values obtained are comparable with

counting range for this example (519 positives for a sampled volume of 15.6 microliters) was 208,000 CFU/ml.

Example 7

Detection and Enumeration of Microorganisms

(Method Utilizing a Plurality of Coated Microcompartments)

This example demonstrates the method wherein nutrient and indicator are incorporated into the microcompartments of the film prior to inoculation with test sample.

VRB media containing MUG fluorescent indicator was prepared as described in Example 3. An excess of this solution was applied to the surface of a film with the microcompartment pattern and geometry described in Example 3. The solution was distributed into the microcompartments by knife coating the solution over the surface of the film. The coated film was then dried in an oven at 52°C. An aqueous dilution of *Serratia liquefaciens* was prepared from an overnight culture to an approximate concentration of about 50 CFU/ml (Butterfield's buffer, Fisher Scientific). A sample of this solution (300 µl) was applied to the nutrient-coated film using the method of Example 3 to fill 420 microcompartments. The sample was incubated overnight at 37°C. Thirty-six fluorescent microcompartments were observed, corresponding to a calculated MPN of 39 (130/ml).

Example 8

Detection and Enumeration of Microorganisms

(Detection Using pH Indicator and Nutrient

Incorporated into Microcompartments)

This example demonstrates absorbance-based detection using an indicator that monitors the pH of the media.

VRB media containing the pH indicator phenol red (1 mg/ml, Sigma Chemical Company) was prepared as described in Example 3. This solution was incorporated into the microcompartments of a film as described in Example 7. An aqueous dilution (Butterfield's buffer, Fisher Scientific) of *Serratia liquefaciens* (approximately 50 CFU/ml) was applied to the film as described in Example 3

the subsequent six dilutions corresponding to volumes of 0.5, 1, 3, 14, 20, and 50 µl. Using this procedure, each microcompartment was filled with diluent containing 2.5 ng of enzyme in 0.25 mM of indicator. A background sample having microcompartments containing only indicator was also prepared.

After filing the microcompartments, the sample was placed in a covered petri dish and sealed with tape to prevent evaporation. The dish was placed inside an ultraviolet illumination and imaging device (UltraLum Corporation, 365 nm). CCD images were stored at the time intervals shown in Figure 4. Fluorescence intensity values for each time point were obtained by averaging 4 pixels at the center of each microcompartment. Final values were obtained by averaging two duplicate experiments.

Figure 4 shows (1) given the same number of enzyme molecules in each well, the kinetics of the reaction are significantly enhanced in the smaller microcompartments, and (2) the fluorescent signal for the detection system (CCD in this case) is enhanced in the smaller microcompartments. To illustrate this effect, the background fluorescence of a microcompartment containing only indicator (no enzyme) is plotted on the graph. On a per-pixel basis, the signal is considerably higher (saturated for the smaller volumes) in the smaller microcompartments than in the larger microcompartments. Note that at the 2-hour time point the 50-µl intensity is 2.3x over background while the 1-µl value is 9.8 x higher. Both the effects of enhanced reaction kinetics and enhanced fluorescence signal led to increasingly more rapid detection as the size of the microcompartments was decreased.

Example 10

Enhanced Microorganism Detection Using Microcompartments (Bacteria + Fluorescent Indicator)

In this example, the same number of bacteria (~5000 CFU) was placed in a plurality of microcompartments ranging in size from 1 to 50 µl. Each microcompartment contained the same concentration of fluorescent indicator (0.25 mM) in a nutrient growth media. The production of fluorescence resulting from

Example 11

Detection and Enumeration of Microorganisms

(Method Utilizing a Plurality of Microchannels)

The feasibility of utilizing film culture devices containing a plurality of covered microchannels to detect and enumerate bacteria was demonstrated in Section A (Single-Layer Film Culture Device) and Section B (Media-Coated Single-Layer Film Culture Device) of this Example. The construction and inoculation of film culture devices containing multivolume "sets" of covered microchannels and containing multilayer film structures are described in Section C and Section D, respectively, of this Example.

A. Single-Layer Film Culture Device

Embossed film containing parallel V-groove microchannels was prepared as described in Example 1D and in U.S. Patent 5,514,120. The resulting film was covered with a silicone PSA/PE top film (Example 1D), thereby creating a series of parallel, covered microchannels having a triangular cross section with a base of approximately 0.6 mm and a height of approximately 0.75 mm. A flat "land area" of approximately 0.3 mm separated each microchannel, and provided an attachment surface for the cover film. The covered films were cut into 2-cm tall x 5-cm wide strips with each strip (film culture device) containing 50 parallel, 2-cm long microchannels. Each channel had a volume of approximately 5 µl (total volume sampled approximately 250 µl). An overnight broth culture of *E. coli* ATCC 51813 was serially diluted into VRB media (Example 3), containing phenol red (0.5 mg/ml). The dilutions were prepared to the following approximate bacterial concentrations (CFU/ml): 10,000; 1,000; 100; and 10. One edge of the embossed film culture device was dipped into the sample and the fluid allowed to wick into the microchannels by way of capillary action. The top edge of the device was then sealed by dipping into melted paraffin to slow evaporation during inoculation. The bottom edge was left open. The samples were incubated overnight at 37 °C inside a humidified petri dish and then observed for red to yellow color changes. A yellow color along an individual channel indicated acid production from bacteria growth (glucose fermentation) within the channel. At the 10,000 CFU/ml and 1,000

Table 11a
Single-Layer Film Culture Device Containing Sets of Microchannels

Set	Strip Width (cm)	Microchannels (MC) Shape and Dimensions	MC Volume (µl)	Total Volume (ml) (30 MC/Strip) (2 Strips/Set)	Counting Range (CFU/ml)
S1	2	Rectangular: 1.75-mm wide 0.65-mm tall	20	1.2	0.8 - 205
S2	1	Rectangular: 0.5-mm wide 0.4-mm tall	2	0.12	7 - 1708
S3	0.8	Triangular: 0.13-mm height 0.4-mm base	0.2	0.012	70 - 17,083

The final single-layer film culture device was assembled by adhering two strips of each volume set (30 microchannels per strip) adjacent to each other at the base of a square petri dish ("Integrid" 100 X 15 mm, Becton Dickenson, Lincoln Park NJ). The strips were attached using transfer tape (Scotch 300LSE Hi Strength Adhesive, 3M Co.) and placed approximately 2 mm apart.

The device was inoculated using a solution containing a food coloring dye to provide contrast. A transfer pipette was used to place the test solution in the "gutter area" between each set of strips. By tipping the device, fluid drained down the "gutter area" and filled the open-ended microchannels (positioned perpendicular to the "gutter area") by capillary action. Excess solution was contained by a strip of paper towel placed at the base of the device. By using the device of this example (60 microchannels per set) and the MPN formula outlined in Example 3, counting ranges for each of the three sets were calculated and are provided in Table 11a.

This example serves to demonstrate that a single-layer film culture device containing sets of microchannels can provide the basis for a bacterial enumeration test that is both highly sensitive and covers a very broad counting range.

D. Multilayer Film Culture Devices

Multilayer film structures were constructed in order to increase both the total volume of liquid sampled and the number of individual enclosed microchannels in the culture device. Two constructions were prepared by laminating together single-layer embossed films and are described in Table 11b. Single-layer films used in multilayer construction D1 contained parallel microchannels having a square cross section with sides approximately 0.2 mm x 0.2 mm. Each microchannel was spaced approximately 0.1 mm apart. Single-layer films were cut into strips 1.5-cm wide x 1-cm tall. A thin layer of adhesive (RD 1273, 3M Co.) was applied to the back of each strip, and the strips were stacked together to form a multilayer

What is claimed is:

1. A method for detecting a microorganism in a liquid test sample, comprising

a) distributing microvolumes of said sample to a plurality of microcompartments of a culture device;

b) incubating said culture device for a time sufficient to permit at least one cell division cycle of said microorganism; and

c) detecting the presence or absence of said microorganism in said microcompartments.

2. The method of claim 1, further comprising the step of quantitating said microorganisms.

3. The method of claim 2, wherein said quantitating comprises determining a most probable number of said microorganisms in said sample.

4. The method of claim 2, wherein said quantitating comprises visually observing said microorganisms.

5. The method of claim 2, wherein said quantitating comprises enumerating said microorganisms in each said microcompartment of said device.

6. The method of claim 1, wherein said microcompartments have reagents coated thereon.

7. The method of claim 6, wherein said coating comprises nutrient medium.

8. An assay device for conducting the method of claim 1, comprising

a) a substrate,

b) said substrate having a plurality of microcompartments therein, said microcompartments each having a top and a bottom surface,

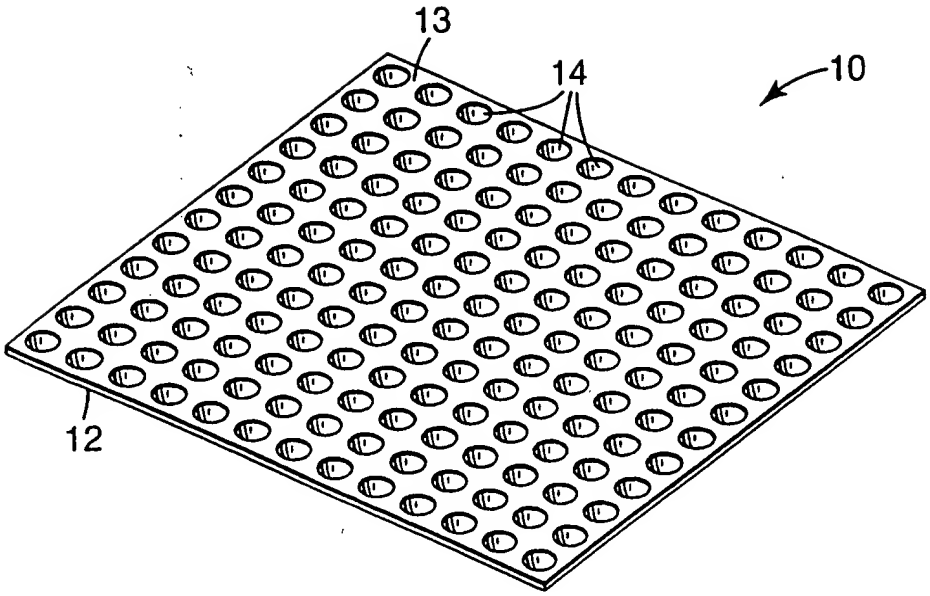

Fig. 1

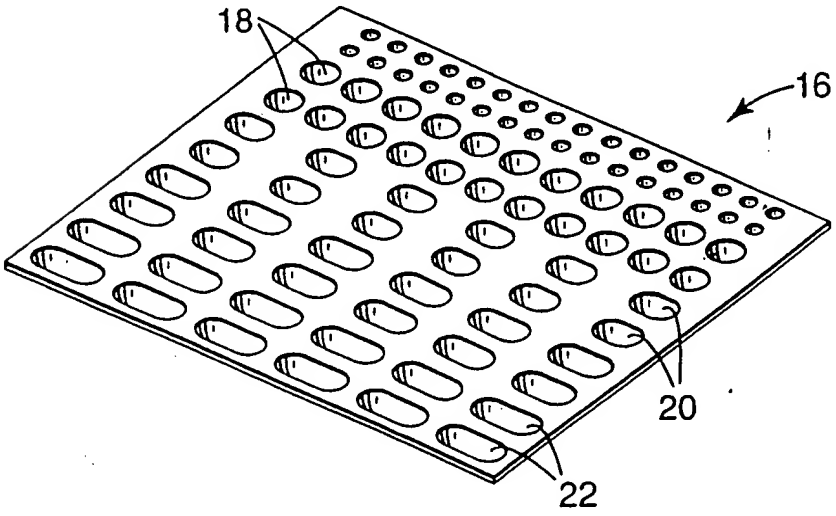

Fig. 2

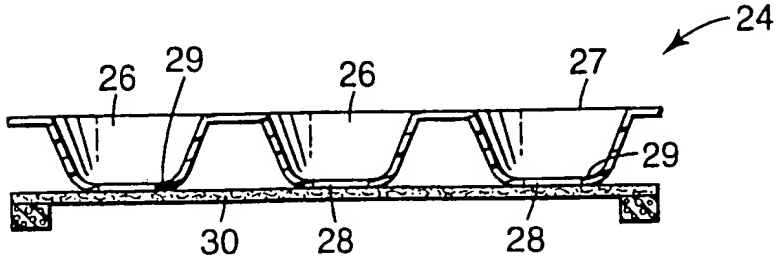

Fig. 3

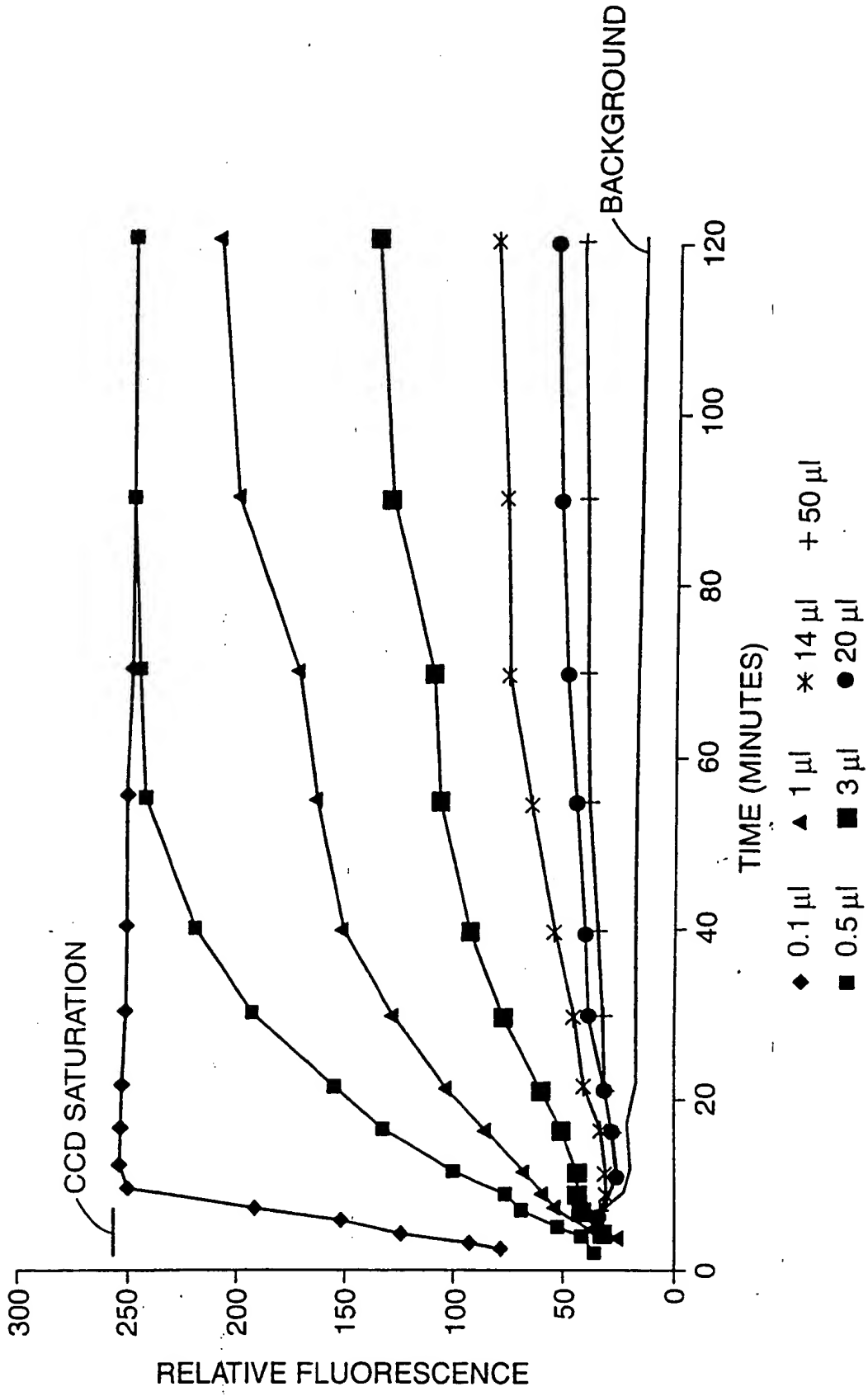
BACKGROUND
CCD SATURATION
RELATIVE FLUORESCENCE
TIME (MINUTES)
0
20
40
60
80
100
120
0
50
100
150
200
250
300
◆ 0.1 µl
▲ 1 µl
✱ 14 µl
+ 50 µl
■ 0.5 µl
■ 3 µl
● 20 µl

Fig. 4

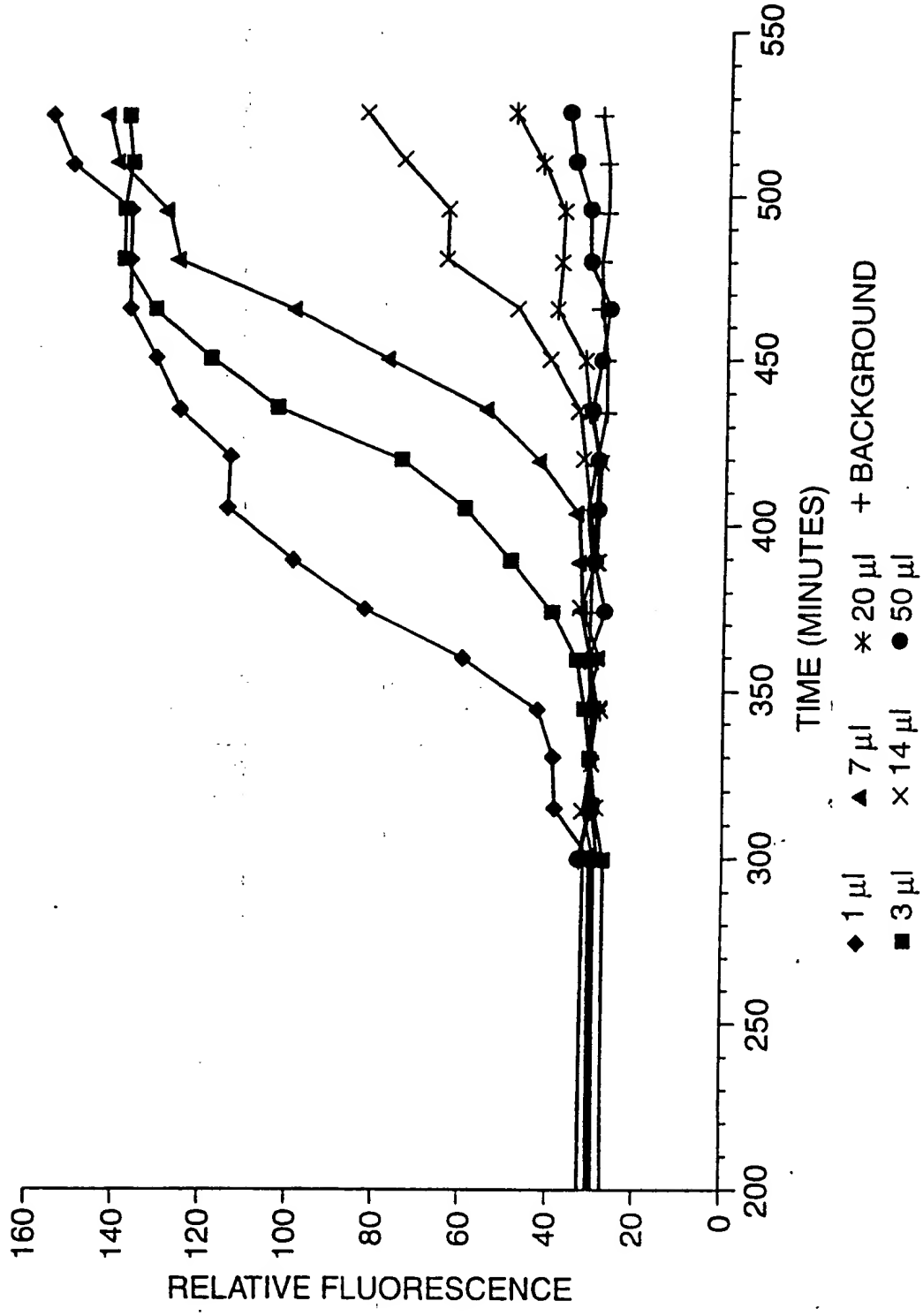
RELATIVE FLUORESCENCE
160
140
120
100
80
60
40
20
0
200
250
300
350
400
450
500
550
TIME (MINUTES)
◆ 1 µl
▲ 7 µl
✳ 20 µl
+ BACKGROUND
■ 3 µl
× 14 µl
● 50 µl

Fig. 5

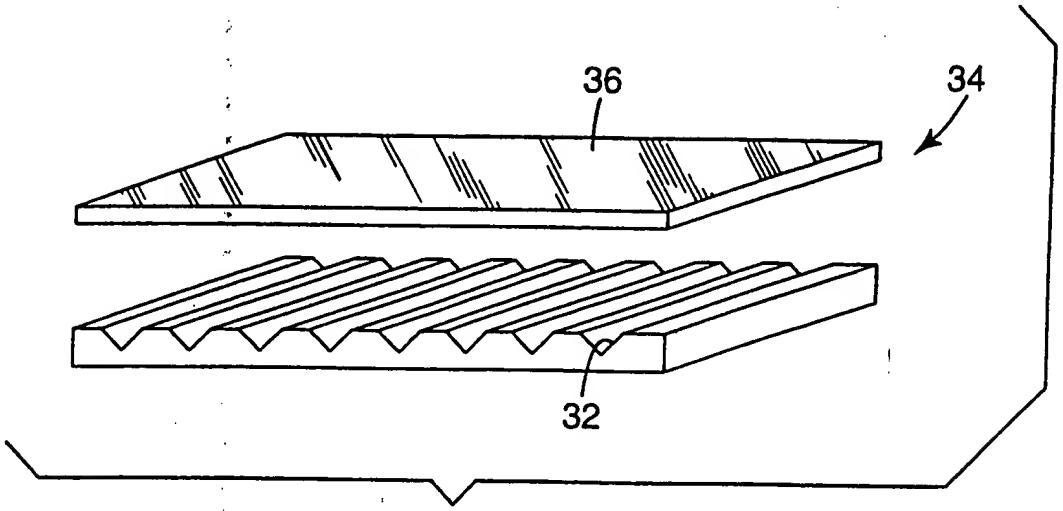

Fig. 6

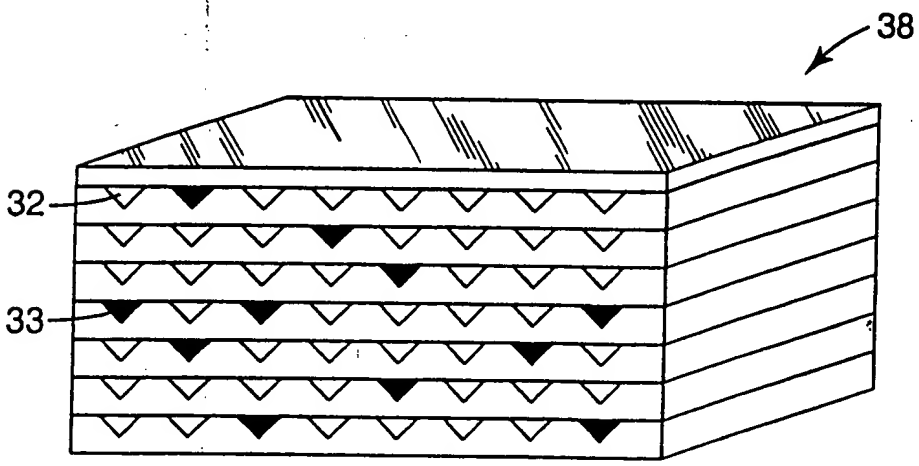

Fig. 7

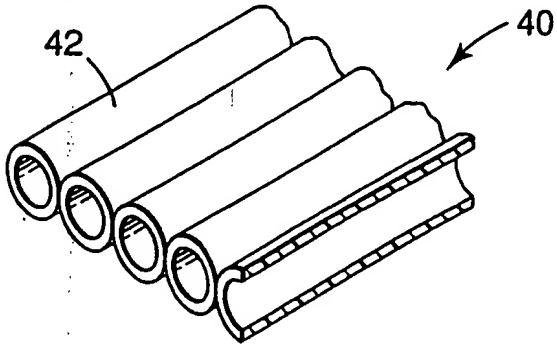

Fig. 8

INTERNATIONAL SEARCH REPORT

Inter ɔnal Application No
PCT/US 98/15575

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/02

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12M C12Q B01L B01D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EP 0 834 729 A (BECTON DICKINSON CO) 8 April 1998 see claims 1-6 see column 3, line 40 - line 56 see column 8, line 15 - line 34 ---	1-10
X	EP 0 496 200 A (BECTON DICKINSON CO) 29 July 1992 see claims see page 6, line 35 - line 44 see example 1 ---	1-10
P,X	WO 98 31466 A (CORNING INC) 23 July 1998 see claims see page 6, line 18 - line 28 see figures 1-3 --- -/--	1-10

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 November 1998	03/12/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Routledge, B

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